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Impact of glycosylation on the immunogenicity of a DNA-based influenza H5 HA vaccine

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Abstract

Avian H5N1 influenza viruses isolated from humans in Hong Kong in 1997 were divided into two antigenic groups based on the presence or absence of a potential glycosylation site at amino acid residues 154–156 in the HA1 region of the viral hemagglutinin (HA) surface glycoprotein. To assess the impact of glycosylation on the immunogenicity of an HA-expressing DNA vaccine, a series of plasmid vaccine constructs that differed in the presence of potential glycosylation sites at amino acid residues 154–156, 165–167, and 286–288 were used to immunize BALB/c mice. Postvaccination serum IgG, hemagglutination inhibition, and neutralizing antibody titers as well as the morbidity and mortality following a lethal H5N1 viral challenge did not vary significantly among any of the experimental groups. We conclude that the glycosylation pattern of the influenza virus HA1 domain has little impact on the murine antibody response raised to a DNA vaccine encoding the H5 HA, thereby minimizing the concern that the pattern of glycosylation sites encoded by the vaccine match those of closely related H5 viruses.
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Keywords: H5N1; Avian influenza; DNA vaccines; Hemagglutinin; Glycosylation

Introduction

The hemagglutinin (HA) is the major surface glycoprotein of the influenza virus that mediates the attachment and penetration of the virus into host cells. The HA is produced as a polyprotein that is proteolytically cleaved into two subunits, HA1 and HA2, which are held together by a single disulfide bond. The HA2 anchors the molecule into the lipid membrane and is responsible for the oligomerization of the protein into its trimeric form. HA1 forms the globular head of the molecule, containing the receptor binding site and conformationally dependent epitopes that are the primary targets for neutralizing antibodies (Dowdle et al., 1974; Kilbourne and Kehoe, 1975; Webster et al., 1975). The HA is therefore the primary focus of current inactivated influenza virus vaccines. However, continual variation in the HA either through the gradual accumulation of point mutations (antigenic drift) or through occasional acquisition of a novel HA gene (antigenic shift) demands the annual reformulation of influenza vaccines (Caton et al., 1982; Raymond et al., 1986; Skehel et al., 1984; Wiley and Skehel, 1987; Wiley et al., 1981; Wilson et al., 1983).

The HA undergoes posttranslational, host-cell-dependent glycosylation. The main purpose of glycosylation on the HA is to aid in correct folding of the molecule and in the formation of the HA trimer. Although all HA molecules are glycosylated, with one exception, the location and number of potential glycosylation sites are not conserved among HAs of different strains and subtypes (Keil et al., 1984; Wilson et al., 1983). The number of sites varies between 5 and 11 in different influenza A strains with general conser-

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vation of the glycosylation sequence occurring around residues 20–22 in HA1. The potential glycosylation sites are scattered throughout the HA protein, but tend to cluster around the antigenic sites in HA1 (Matrosovich et al., 1999; Wilson and Cox, 1990). The presence or absence of a given host-derived carbohydrate moiety can modulate the antigenicity of the HA molecule by preventing the binding of neutralizing antibody or by masking epitopes recognized by $CD4^+$ T cells (Skehel et al., 1984; Wiley and Skehel, 1987). This has also been shown recently with the glycosylation of the envelope glycoprotein of the human immunodeficiency virus (Quinones-Kochs et al., 2002). Thus, the acquisition of carbohydrate moieties provides a mechanism for evasion from the immune system.

In 1997, avian influenza A (H5N1) viruses were identified as the causative agent of 18 cases of human respiratory illness, including six deaths in Hong Kong (Center, 1997, 1998; Subbarao et al., 1998). Human H5N1 isolates were divided into two distinct groups based on antigenic differences when tested against reference postinfection ferret antisera by the hemagglutination-inhibition (HI) assay (Bender et al., 1999). In addition, the two groups differed genetically due to the absence (Group A) or presence (Group B) of a potential glycosylation site (N-X-S/T, X ≠ P) at amino acids (aa) 154–156 in HA1, located at the tip of the globular head domain of HA.

The ability of avian H5N1 viruses to infect and cause severe respiratory illness in humans highlighted the pandemic potential of this virus and the need for an effective vaccine. Traditional strategies have, as yet, failed to produce a suitably immunogenic vaccine against the H5N1 viruses (Lu et al., 1999; Nicholson et al., 2001; Treanor et al., 2001; Wood et al., 1999). Immunizing with purified DNA is a powerful technique for inducing immune responses. Comparative studies of conventional and influenza HA-based DNA vaccines have shown that DNA vaccination is equivalent to immunization with a live virus vaccine in protecting mice from challenge and both vaccines were superior to a subunit vaccine (Boyle et al., 1996; Justewicz et al., 1995). While carbohydrate moieties clearly can modulate the antigenicity of HA, little is known about the impact of glycosylation on the quantity or quality of the antibody response to a DNA-expressed HA vaccine. To investigate the impact of glycosylation on the immunogenicity of an H5 HA-expressing DNA vaccine, BALB/c mice were vaccinated with plasmids that were engineered to differ in the presence or absence of potential glycosylation sites in the globular head domain of HA1. Based on the analysis of H5 HA sequences from 1959 to 1997, three potential glycosylation sites (residues 156, 167, and 288) that were not conserved in all H5 strains were chosen since these sites were likely not critical for the structural integrity of the folded protein. However, these sites were conserved in 14 of 15 highly pathogenic H5 viruses in our analysis, suggesting a role for these carbohydrate moieties in viral pathogenesis (Matrosovich et al., 1999). While the crystal structure of the H5 HA has recently been determined (Ha et al., 2001), the antigenic sites on the H5 HA are not yet fully defined. Recent studies have identified two antigenic sites on the H5 HA, using monoclonal antibody escape mutants. Based on this work, the glycosylation site at residue 156 is in or near the antibody-combining site B (Kaverin et al., 2002). By analogy with the H3 HA structure, the glycosylation site at residue 167 would also be in or near antigenic site B, whereas the glycosylation site at residue 288 would be in or near antigenic site C (Wilson and Cox, 1990). Here we show that an HA-expressing DNA vaccine protected animals from challenge with a highly lethal H5N1 virus and that altering the glycosylation pattern on H5 HA did not substantially alter the quantity or quality of the neutralizing antibody response in BALB/c mice.

Results

Generation and expression of plasmids

In preliminary experiments, the entire HA coding sequence from the influenza HK/156 and HK/483 viruses was cloned into the pGA expression vector to raise polyclonal rabbit antisera by gene gun immunization of New Zealand white rabbits with the plasmids. Rabbits inoculated three times with 12 μg of DNA expressing the HA from HK/156 lacking a potential glycosylation site at aa 154–156 in the globular head domain of HA1 elicited higher titers of anti-HA antibodies than the titers from rabbits inoculated with a DNA vaccine expressing the HK/483 HA, which contains a potential glycosylation site at these residues. These results suggested that the extent of glycosylation may impact the immunogenicity of the HA molecule expressed in a DNA vaccine construct.

To investigate the impact of the glycosylation state of the HA on the immunogenicity of this protein, DNA plasmids expressing either the wild-type HK156 or the mutated HA were constructed. The HK156wt DNA plasmid contained N-linked potential glycosylation sites at amino acids 165 and 288, while five additional HA expression plasmids were constructed that expressed the HK/156 HA gene mutated to contain various combinations of N-linked potential glycosylation sites at amino acids 156, 167, or 288 by site-directed mutagenesis (Fig. 1). Each HA gene was cloned into the pGAT0 mammalian expression vector (Fig. 1).

To verify expression of each HA protein, DNA vaccine plasmids were transiently transfected into human 293T cells and the cell lysates were assayed for expressed HA. Each gene product was expressed and detected as a band of 65–70 kDa using polyclonal rabbit antisera raised against the HK156wt HA. We observed an upward or downward shift of the HA bands on the Western blot, indicating an alteration in the size of the expressed protein consistent with the addition (RB536) or deletion (RB537, RB539, RB552,

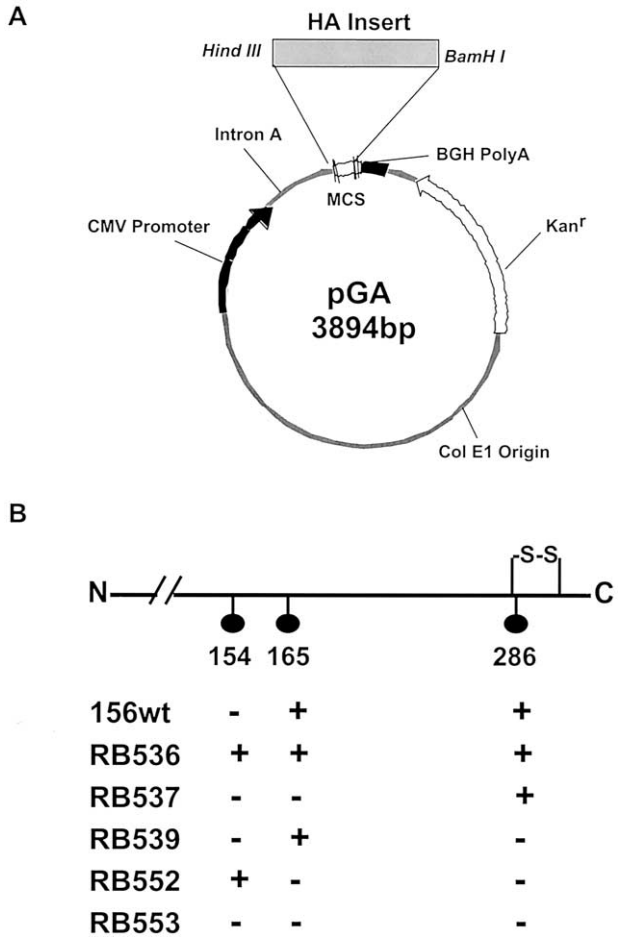

Fig. 1. Schematic representation of vector DNA constructs used for immunizations. (A) The pGAT0 vector contains the cytomegalovirus immediate-early promoter (CMV-IE) plus Intron A (IA) for initiating transcription of eukaryotic inserts and bovine growth hormone polyadenylation signal (BGH poly(A)) for termination of transcription. The vector also contains the CoIE1 origin of replication for prokaryotic replication as well as the Kanamycin resistance gene (Kan^r) for selection in antibiotic media. The lambda T0 terminator has been placed 3′ to the Kan^r to increase the stability of eukaryotic inserts. HA inserts were cloned into the vector using the *Hind*III and *Bam*HI restriction endonuclease sites. (B) Schematic of a truncated H5 HA1 region illustrating the pattern of three potential glycosylation sites altered in the listed vaccine constructs.

RB553) of glycosylation sites, respectively (data not shown).

Antibody responses of mice immunized with HA expressing DNA vaccines

The ability of the HA-expressing DNA vaccines to induce serum ELISA, HI, and neutralizing antibodies to the homologous HK/156 virus was examined. Groups of mice (n = 10) were vaccinated with 1 μg of HK156wt, RB536, RB537, RB539, RB552, or RB553 HA-expressing plasmid or 1 μg of pGAT0 vector with no insert for a negative-control DNA. Four weeks later, the mice were boosted with 1 μg of plasmid DNA. Sera collected 4, 6, 10, and 14 weeks after the first immunization were analyzed for the presence of H5-specific antibodies by ELISA, HI, and neutralization assays.

IgG antibody titers in sera collected 4 weeks after the first inoculation and 2 weeks after the boost were measured using an ELISA with a purified, recombinant HK/156 HA protein to coat the plates. Each of the constructs elicited a substantial H5 HA-specific IgG response after the first inoculation, suggesting that each construct was adequately delivered and expressed in mice. A 10- to 15-fold boost in IgG antibody response was seen with each construct after the second inoculation. There was no significant difference in serum IgG antibody levels raised toward any expressed HA when compared with the HK156wt HA (Fig. 2).

To assess the quantity of functional antibodies elicited by the various HA-expressing constructs, the HI assay, the "gold standard" for serological measurement of an immune response to influenza, was used to detect antibody against homologous HK/156 virus. Because the HI assay was shown previously to be less sensitive for the detection of antibodies induced by avian influenza viruses, we also used the more sensitive microneutralization assay (Rowe et al., 1999). At week 14, the titer of HI antibodies was significantly higher in the group inoculated with RB553, when compared to the HK156wt ($P < 0.05$) (Fig. 2). The microneutralization assay showed that, in general, there was no significant difference in neutralizing antibody titers at week 6. However, at week 14 the neutralizing antibody titer raised by the RB537-expressed HA was significantly lower than that induced by the HK156wt ($P < 0.05$) (Fig. 2). Overall, these results indicate that there was little difference in the ability of each mutated HA to raise functional antibodies when compared to the wild-type HA against a homologous virus.

Specificity of neutralizing antibodies raised against the DNA-expressed HA

Since there was no difference in the relative quantity of neutralizing antibodies raised, we next determined whether differences in glycosylation in HA1 affected the extent of cross-reactivity of the antibody response. Sera collected at week 14 from each vaccine group were pooled and tested for the ability to neutralize closely or more distantly related H5 influenza viruses as well as viruses of other subtypes (H1, H3, and H9) (Table 1). None of the sera from any vaccine group was able to neutralize viruses from the H1, H3, or H9 subtypes or the H5 viruses Ty/Wis or Ck/Scot, which shared $\leq 90\%$ similarity in their amino acid sequence compared to the HK/156 HA (data not shown). The neutralizing antibody titers for the other H5 viruses are shown in Fig. 3. Neutralizing antibody titers against the closely related HK/483 virus were similar to those against the wild-type HK/156 virus. There was a direct correlation between the percentage amino acid homology of the HA and the ability to neutralize virus. As the percentage of

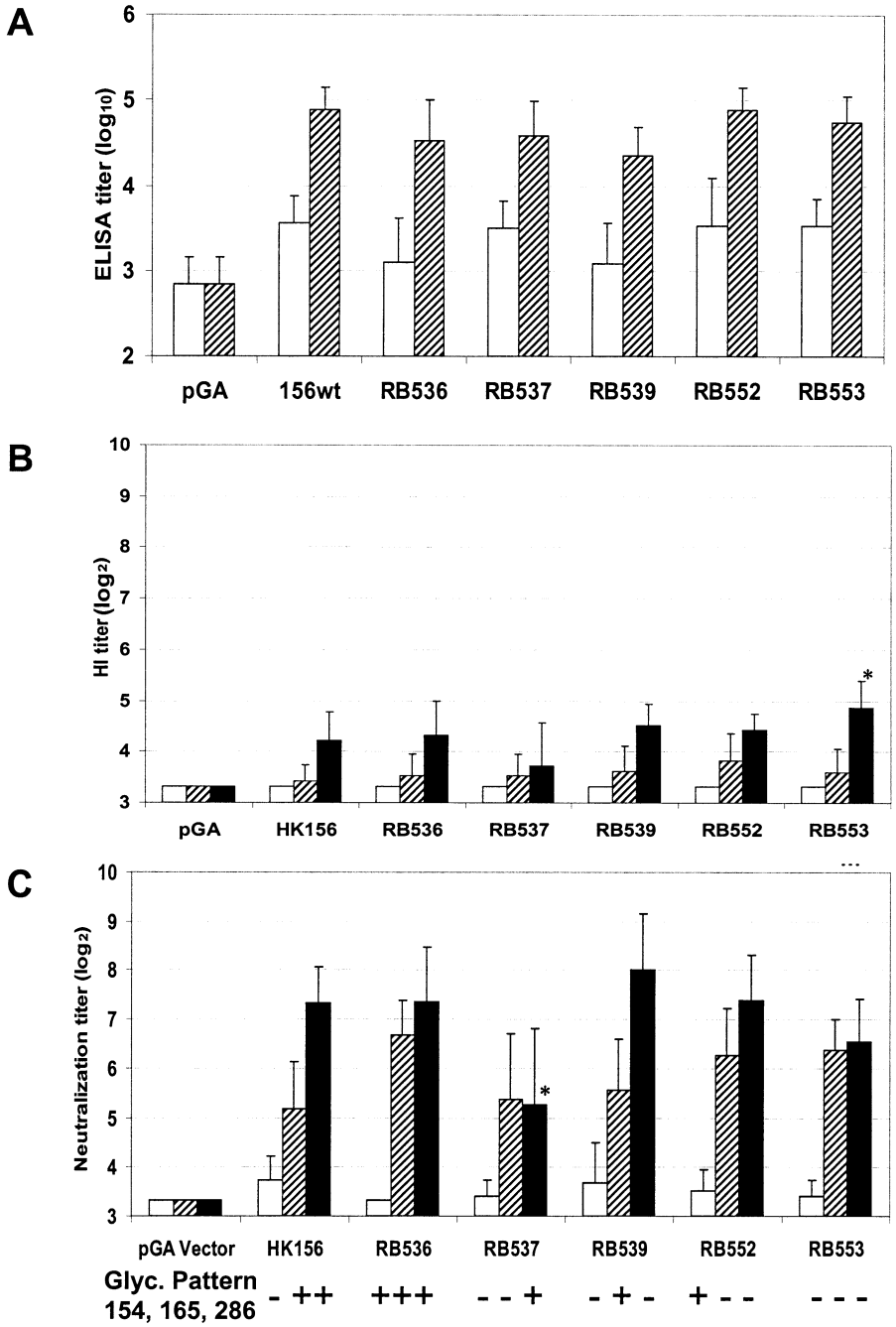

Fig. 2. Antibody responses to HK156 HA-expressing DNA vaccines with different glycosylation patterns. Groups of 10 mice were inoculated on day 0 and at week 4 with 1 μg plasmid DNA via gene gun to the abdominal epidermis. (A) ELISA serum IgG antibody titer to HK/156 rHA. Sera were collected at weeks 4 and 6 to determine IgG antibody levels after one and two inoculations, respectively. IgG titers are expressed as $\log_{10}$ endpoint titers. (B) Hemagglutination-inhibition (HI) antibody titers measured against HK/156 virus in sera collected at weeks 4, 6, and 14. HI titers are expressed as the $\log_2$ value of the reciprocal of the highest dilution of serum inhibiting agglutination of 0.5% turkey erythrocytes by 4 HA units of virus. (C) Neutralizing antibody titers measured against HK/156 virus in sera collected at weeks 4, 6, and 14. Neutralizing titers are expressed as the $\log_2$ value of the reciprocal of the highest dilution of serum neutralizing antibodies inhibiting 100 $TCID_{50}$ of virus growth in MDCK cells. *$P < 0.05$ for experimental group versus HK156 wild-type HA. (Open bars, week 4; shaded bars, week 6; filled bars, week 14.)

amino acid homology decreased, the neutralizing antibodies induced by the constructs lost the ability to neutralize these viruses, regardless of the glycosylation pattern of the HA used as an immunogen. Therefore, neutralizing antibodies induced by the HK/156 DNA vaccines showed minimal differences in their ability to recognize the HA from closely related H5 viruses and did not show any heterosubtypic reactivity.

Table 1
Genetic relatedness of influenza viruses based on amino acid sequence homology of the hemagglutinin (HA)

Virus	Subtype	% homology—HK156 HA
A/Puerto Rico/8/34	H1N1	63.6
A/Sydney/5/97	H3N2	37.8
A/Hong Kong/1073/99	H9N2	49.2
A/Hong Kong/156/97	H5N1	100
A/Hong Kong/483/97	H5N1	98.6
A/Duck/Singapore-Q/F119-3/97	H5N1	94.0
A/Duck/Hong Kong/79	H5N3	93.3
A/Chicken/Scotland/1/59	H5N1	90.6
A/Mallard/Wisconsin/944/92	H5N2	89.9
A/Turkey/Wisconsin/1/68	H5N3	89.4

Protection against influenza in mice immunized with wild-type or nonglycosylated HA-expressing DNA vaccines

To determine the extent of protection provided by each of the HA-expressing plasmids, mice were challenged with the HK/156 virus. Fourteen weeks after the first inoculation, groups of six mice were challenged intranasally with 10 MLD_{50} of HK/156. In addition to the DNA-inoculated mice, a naïve group of mice was infected as a control. Weight loss and survival was monitored daily for 14 days. Fig. 4 presents the data for the individual groups of mice. Substantial weight loss and death were seen in the naïve mouse group and in mice that received the empty vector DNA. The RB537 group was the only group to show a substantial, yet transient weight loss. Both RB536- and RB537-inoculated groups had one mouse succumb to infection on days 7 and 8, respectively. Therefore, all HA-expressing plasmids induced 83 to 100% protection of mice from lethal challenge with H5N1 virus. These results indicate that there was no significant difference in the ability of each DNA construct to protect mice against a lethal homologous challenge.

Discussion

In this study, we have investigated the relative immunogenicity and protective efficacy of DNA vaccines expressing HK/156 HA and containing different glycosylation patterns at three sites within HA1 in mice. The different patterns of glycosylation in the HA1 did not significantly alter the quantity of neutralizing antibodies raised in the mice nor did they affect the cross-reactivity of antibodies for related H5 viruses. Protection of mice against lethal challenge with H5N1 virus was achieved from each DNA-expressing HA vaccine with varying patterns of the glycosylation in the HA1 region. Only one group, RB537, showed a modest reduction in the neutralizing antibody titer, resulting in the death of one mouse, which may reflect a single poor-responder to vaccination. This study confirms

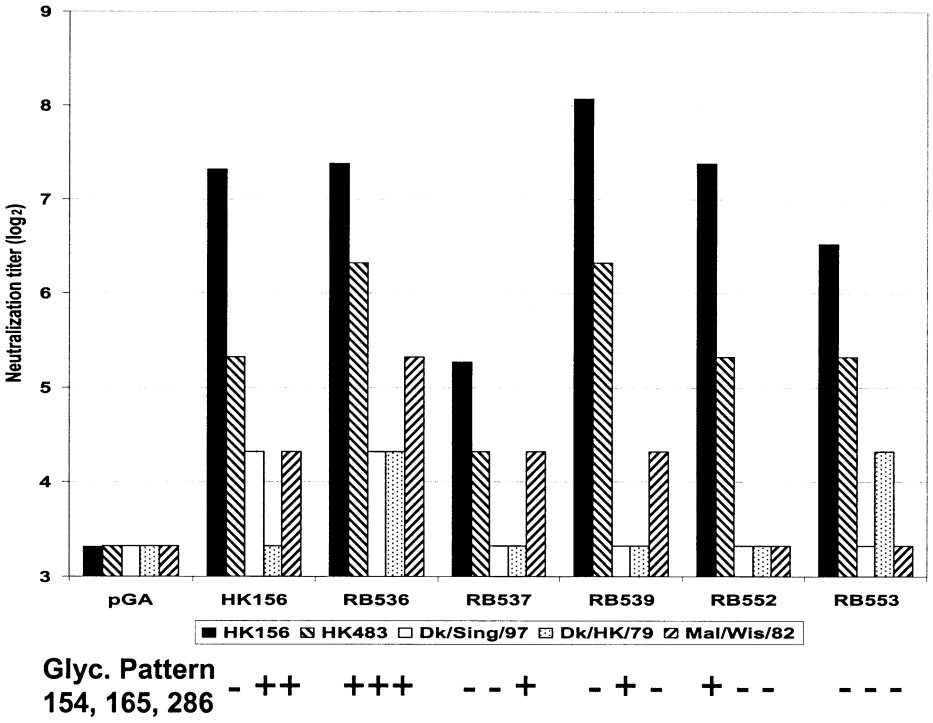

Fig. 3. Neutralizing antibody titers to H5 influenza viruses. Week 14 sera from 10 mice per group were pooled and tested for the ability to neutralize 100 $TCID_{50}$ of closely related H5 influenza viruses. The data are expressed as the $\log_2$ value of the reciprocal of the highest dilution of sera able to neutralize the respective virus.

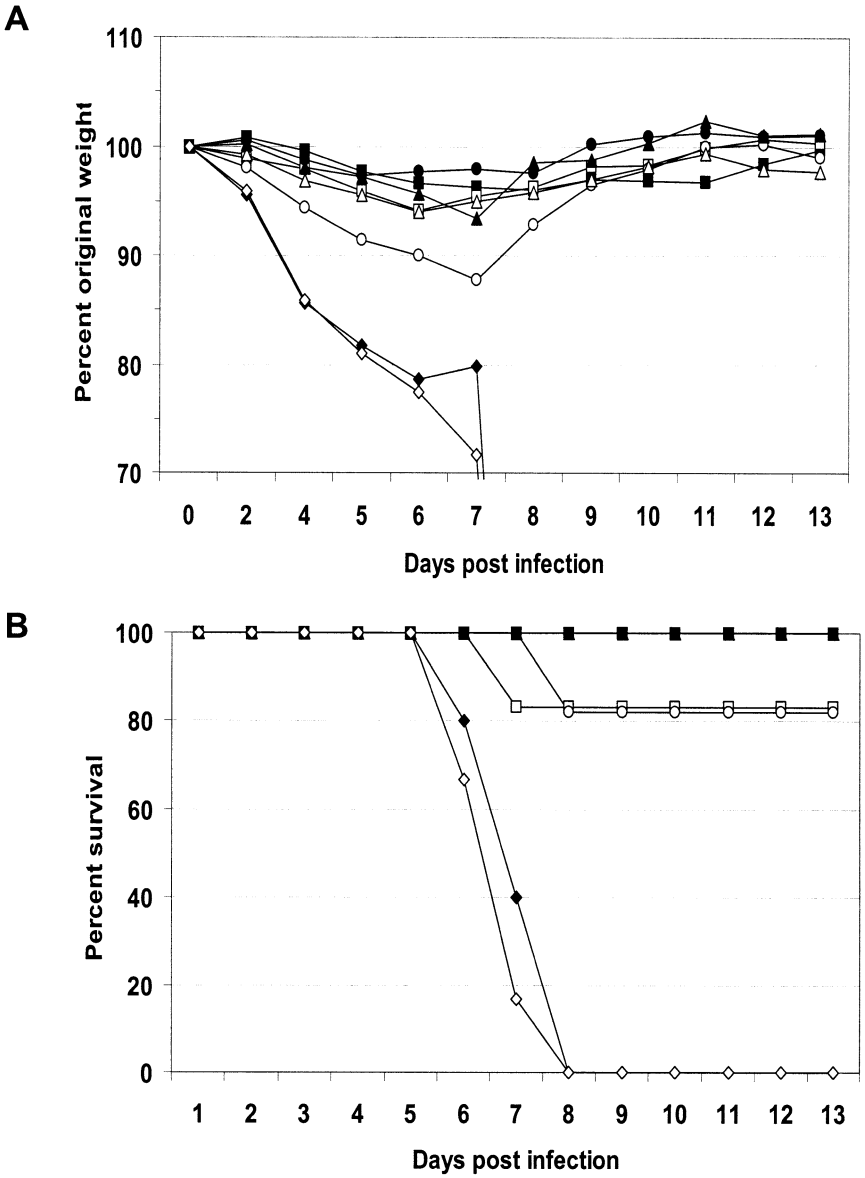

Fig. 4. Weight loss and survival in mice administered HA-expressing DNA vaccines. Groups of six mice were inoculated with 1 μg of plasmid DNA on day 0 and at week 4. Fourteen weeks after the initial inoculation, mice were challenged with 10 LD_{50} of HK/156 virus and monitored for 14 days. (A) Mean weight loss expressed as a percentage of original weight. (B) Percent survival (◆, pGA vector; ■, HK156wt (− + +); □, RB536 (+ + +); ○, RB537 (− − +); ●, RB539 (− + −); △, RB552 (+ − −); ▲, RB553 (− − −); ◇, unvaccinated).

and extends previous results from preclinical studies demonstrating that an H5 HA-expressing DNA vaccine can provide protection from fatal H5N1 disease that was associated with systemic spread and an inflammatory response in the brain of BALB/c mice (Kodihalli et al., 1999).

The avian outbreak of H5N1 viruses in 1997 that caused respiratory disease and death in humans and the subsequent occurrence of an H9N2 infection in humans highlighted the potential threat of such novel avian subtypes for further infections among susceptible humans. Since that time, H5N1 viruses have reemerged in live poultry markets in Hong Kong several times, while H9N2 viruses remain widespread in domestic poultry throughout Asia (Cauthen et al., 2000; Guan et al., 2000; Webster et al., 2002). The potential for novel avian subtypes to reassort with human viruses and create a pandemic strain necessitates the evaluation of current and alternative vaccine approaches to provide protection in such an event. Several approaches have been used in mice to generate an H5N1 vaccine; using traditional inactivated vaccines based on the highly pathogenic H5N1 virus itself, an antigenically related apathogenic H5N3 virus, or a baculovirus-produced recombinant H5 HA protein. Although the inactivated H5N1 vaccine was immunogenic and protected mice from a lethal H5N1 challenge (Wood et al., 1999), the lethality of the virus for chicken embryos, resulting in suboptimal virus titers (Takada et al., 1999), and the need for BSL3+ containment for vaccine production, limits the utility of this approach (Wood et al., 1999). The use of

the surrogate, apathogenic virus, Dk/Sing, as a traditional vaccine overcame the need for containment facilities. However, in both mice and humans the immunogenicity of two doses of inactivated Dk/Sing was suboptimal and required the use of adjuvants for improved immunogenicity (Lu et al., 1999; Nicholson et al., 2001; Wood et al., 1999). Likewise, the baculovirus recombinant H5 HA vaccine induced suboptimal immunity in humans (Treanor et al., 2001). In light of these results, it is important to examine the efficacy of alternative vaccine strategies such as DNA vaccines for H5 viruses. Studies on preclinical efficacy showed that DNA expression vectors encoding the influenza HA, plus NP and M1, in ferrets and nonhuman primates were as effective as conventional inactivated vaccines in reducing viral shedding (Donnelly et al., 1995). DNA vaccines expressing the HA of HK/156 also protected mice from a lethal challenge of homologous virus, even in the absence of detectable HI antibody titers (Kodihalli et al., 1999). In addition, it has been shown that DNA vaccines based on conserved influenza proteins, NP and M1, from the PR8 virus could provide heterosubtypic protection and control the infection of mice with the mildly pathogenic HK/156 virus (Epstein et al., 2002).

DNA vaccines offer two main advantages for the development of a pandemic influenza vaccine. First, a DNA vaccine can be rapidly prepared simply by cloning the relevant HA, with or without other viral genes, into an appropriate plasmid-based expression vector as soon as a novel virus with pandemic potential is identified. In cases where a highly pathogenic avian virus is detected, DNA vaccines overcome the limitations of growth in embryonated eggs and the need for containment facilities. However, to date, DNA vaccines expressing influenza HA have failed to elicit substantial antibody responses in humans, although only intramuscular delivery has been evaluated. Particle-mediated or gene gun delivery modalities may well improve DNA vaccine immunogenicity in humans as has been shown in animal models including nonhuman primates (Kent et al., 2001; Lodmell et al., 2001).

This study has demonstrated that differences in number of nonconserved glycosylation sites within HA1 of a given subtype of influenza should not affect the ability to elicit neutralizing antibodies of DNA-expressed proteins. Furthermore, differences in HA1 glycosylation patterns also had no effect on the degree of antibody cross-reactivity between related strains. We conclude that DNA vaccines should be evaluated further as a preventative strategy against pandemic influenza.

Materials and methods

Viruses

The influenza viruses used in this study were A/Hong Kong/156/97 (HK/156), A/Hong Kong/483/97 (HK/483), and A/Chicken/Scotland/1/59 (Ck/Scot) (all H5N1); A/Duck/Hong Kong/79 (Dk/HK), A/Duck/Singapore-Q/F119-3/97 (Dk/Sing), and A/Turkey/Wisconsin/1/68 (Ty/Wis) (all H5N3); A/Mallard/Wisconsin/944/82 (Mal/Wis) (H5N2); A/Puerto Rico/8/34 (PR/8) (H1N1); A/Aichi/2/68 (Aichi) (H3N2); and A/Hong Kong/1073/99 (H9N2). A U.S. Department of Agriculture permit was obtained before work with avian influenza viruses was begun. Virus stocks were propagated in the allantoic cavities of 10-day-old embryonated hen eggs under conditions that were found to be optimal for virus replication (Lu et al., 1999). Virus stocks were stored at −70°C until use.

Due to the potential risk to humans and poultry, all experiments using infectious pathogenic avian viruses, including animal challenges, were conducted using BSL3+ containment procedures (Richmond and McKinney, 1993). Investigators were required to wear appropriate respirator equipment (RACAL Health and Safety, Inc., Frederick, MD).

Plasmids and mutagenesis

A full-length cDNA copy of the HA gene of HK/156 was cloned into a pUC19 vector. The full-length HA gene from this clone was obtained by digestion with *Hin*dIII-*Bam*HI restriction enzymes, and the resulting fragments were ligated into a pGAT0 mammalian expression vector that has been previously described (Ross et al., 2000). This plasmid construct was designated HK156wt. Primers used for mutagenesis of the HK156wt HA gene to create the plasmids RB536, RB537, RB539, RB552, and RB553 were S288A: F-gcgataaactctgctatgccattccac, R-gtggaatggcatagcagaggttatcgc; A156T: F-caaaaagaacagtacatacccaacaataaa, R-ctttatgttgggtatgtactgttctttttg; and T167A F-ggagctacaataatgccaaccaagaag, R-cttcttggttgttggcattattgtagctcc. Mutations were made using a 16-cycle PCR reaction with the QuickChange Mutagenesis Kit (Life Sciences, Carlsbad, CA) following the manufacturer's guidelines. The expression plasmids along with their specific glycosylation pattern at the three targeted sites are listed in Fig. 1.

The plasmids were amplified in the DH5α *Escherichia coli* strain, purified using anion-exchange resin columns (Qiagen, Valencia, CA), and stored at −20°C in dH_2O. Plasmids were verified to contain the correct size gene insert of 1.7 kb by digestion with *Hind*III-*Bam*HI restriction enzymes and gel electrophoresis. The constructs were also fully sequenced through the HA-insert region to confirm that only the mutations of interest were made. Purity of DNA preparations was determined by optical density readings at 260 and 280 nm.

Transfections and expression analysis

The human embryonic kidney cell line, 293T (5×10^5 cells/transfection), was transfected with 2 μg of DNA using 12% Lipofectamine according to manufacturer's (Life Technologies, Grand Island, NY) guidelines in 1 ml of DMEM without fetal calf serum (FCS) in one well of a six-well plate. After overnight incubation, the transfection mixture was aspirated and replaced with 2 ml of fresh

DMEM containing 10% FCS plus antibiotics. Cells were incubated for an additional 48 h at 37°C in 5% CO_2. Cell lysates were collected in 300 μl of RIPA lysis buffer (0.05M Tris–HCI, pH 8.0, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 M NaCI) and stored at −70°C.

For Western blot analysis, 30 μg of total protein from each cell lysate was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA) and loaded onto a 10% SDS–polyacrylamide gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and incubated with a 1:2000 dilution of polyclonal rabbit anti-H5 HA antisera in PBS containing 0.1% Tween 20 and 1% nonfat dry milk. After extensive washing of the membrane, bound rabbit antibodies were detected using a 1:2000 dilution of horseradish peroxidase conjugated goat anti-rabbit antiserum and enhanced chemiluminescence (Amersham, Piscataway, NJ).

Mice and DNA immunizations

Female BALB/c mice, 6 to 8 weeks old (Charles River Laboratories, Wilmington, MA), were used in all experiments. Mice, housed in microisolator units and allowed free access to food and water, were cared for under USDA guidelines for laboratory animals under an approved institutional protocol. Mice were anesthetized with 0.03–0.04 ml of a 5:1 mixture of ketamine HCI (100 mg/ml) and xylazine (20 mg/ml). Gene gun immunizations were performed on shaved abdominal skin using the previously described hand-held Accell gene delivery system (Haynes et al., 1994; Pertmer et al., 1995; Pertmer and Robinson, 1999). Mice were immunized at week 0 and boosted at week 4 with 1 μg of DNA containing 0.5 μg of DNA per 0.5 mg of −1-μm gold beads (DeGussa-Huls Corp., Ridgefield Park, NJ) at a helium pressure setting of 400 psi.

Serologic testing

Postvaccination sera from blood samples (weeks 4, 6, 10, and 14) collected from the orbital plexus were treated with receptor-destroying enzyme from *Vibrio cholerae* (Denka-Seiken, Tokyo, Japan) as previously described (Katz et al., 1997) before testing for the presence of HI antibody by standard methods (Kendal et al., 1982). Individual mice were considered to have responded to vaccination if serum HI titers were ≥40. Titers of neutralizing antibody were determined as previously described (Harmon et al., 1988). Neutralization titers are expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 $TCID_{50}$ of virus. Standard indirect ELISAs were performed to assess anti-HA specific IgG levels in postvaccination sera using 1 μg/ml of purified recombinant HK/156 HA protein (Protein Sciences, Meriden, CT) to coat plates. Endpoint titers are expressed as the reciprocal of the endpoint dilution of sample with an OD_{490} reading three times the mean OD_{490} of wells that lacked primary antibody.

Influenza virus challenge

Fourteen weeks after the initial inoculation, mice were lightly anesthetized with CO_2 and challenged with 10 MLD_{50} of influenza virus HK/156 (H5N1) by intranasal delivery of 50 μl of allantoic fluid diluted in PBS. This method leads to infection of both the upper and lower respiratory tract and is lethal to 100% of nonimmunized mice. Mice were monitored daily for both weight loss and survival. Data are presented as the average percentage of the prechallenge weight.

Statistics

Statistical analyses were performed using the Student's *t*-test to compare each modified HA to the HK156wt construct. Statistical significance was considered when the *P* value was ≤0.05.

Acknowledgments

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